TITLE OF THE INVENTION

TRIMERIC AND POLYMERIC ALKALOIDS

CROSS-REFERENCE TO RELATEDAPPLICATION

This application claims priority from a provisional application which was filed in the United States on July 31, 1998, having Serial No. 60/095,000, incorporated herein by reference.

BACKGROUND OF THE INVENTION

The present invention relates to extracts of disease-resistant *Catharanthus* plants, to trimeric and polymeric alkaloids produced by these plants and to the use of the extracts, trimeric alkaloids and polymeric alkaloids as medicinals and anti-biological agents.

The publications and other materials used herein to illuminate the background of the invention, and in particular, cases to provide additional details respecting the practice, are incorporated by reference, and for convenience are referenced in the following text by author and date and are listed alphabetically by author in the appended list of references.

Catharanthus roseus (L.) G. Don, also called periwinkle, originates from Madagascar and belongs to Apocynaceae family. *Catharanthus* spp. are well known for their production of indole alkaloids (Farnsworth, 1961; Taylor and Farnsworth, 1975). *Catharanthus* is one of the most extensively studied medicinal plants. Since 1950, well over 1200 scientific publications, including about 90 patents dealing with this plant have appeared (Moreno et al., 1995). *Catharanthus roseus* produces a large variety of monomeric indole alkaloids (Lounasmaa and Galambous, 1989). In addition to the 100 or more known natural monomers, *Catharanthus roseus* also produces dimeric indole alkaloids including naturally-occurring vinblastine and vincristine, two medically important anti-tumor agents used in the chemical treatment of human cancers.

As a consequence of the discovery of anti-cancer activity associated with *Catharanthus* alkaloids (Svoboda and Blake, 1975), numerous studies on the pharmacological activity of these compounds have occurred. Within the monomeric group of alkaloids, none have been shown to have significant anti-cancer activity, and only two (ajmalicine as an anti-hypertensive agent and serpentine as a sedative) are of minor commercial value (Moreno et al., 1995). In contrast, essentially all of the natural dimers appear to have at least some associated anti-cancer activity

research on cell biology of *Catharanthus roseus* for well over 30 years. Despite such rigorous

research effort aimed at cell or tissue culture (DiCosmo and Misawa, 1995) or in vitro synthesis (Kutney, 1990; U.S. Patent No. 5,047,528), massive quantities of whole plant parts (especially leaves) are still the commercial source for these bisindole alkaloids.

Dimeric indole alkaloids in *Catharanthus* are formed by *in vivo* condensation of vindoline and catharanthine monomers. Meijer et al. (1993) and Sottomayor et al. (1997) have reviewed enzymatic aspects of catharanthine and vindoline biosynthesis in *Catharanthus* leading to coupling to form anhydrovinblastine, the precursor of other bisindoles including vinblastine, vincristine, leurosine and catharine. While the monomer catharanthine is found in all parts of *Catharanthus roseus* plants as well as in root or shoot cultures, vindoline and the bisindole alkaloids accumulate only in whole green parts, including shoot cultures. The biosynthesis and accumulation of vindoline in the intact plant is controlled by tissue-specific, developmentally regulated and light-dependent factors (Aerts and DeLuca, 1992; St-Pierre and DeLuca, 1995). Dependence on *in planta* synthesis of vindoline and the general rarity of bisindoles in whole leaves (.003% dry wt.; Sottomayor et al., 1996), contributes to the high cost of the bisindole chemotherapeutics.

All of the known naturally occurring bisindoles, including vinblastine and vincristine, are representable by the formula shown in Figure 1. The upper heterocyclic component is the catharanthine monomer and the lower heterocyclic monomer is the vindoline monomer. In Figure 1, numbering conventions of previous U.S. patents 3,932,417; 4,303,584; 4,199,504; 4,203,898; 4,375,432; 4,479,957 have been followed. In the formula of Figure 1, vinblastine (VB) is represented by R being methyl and vincristine (VC) is represented by R being formyl. It should be noted that other numbering conventions (including IUPAC carbon numbering) are frequently encountered in the literature; variance in numbering nomenclature greatly contributes to confusion in comparisons of alkaloid ring structures. Figure 1 has retained the numbering conventions of early Eli Lilly patents to be consistent with the existing large body of bisindole alkaloid literature. This numbering system will be referred to herein with reference to, e.g., unsaturation or saturation.

Even though the structural difference between vinblastine (R = CH_3) and vincristine (R = CHO) is minor, the compounds exhibit substantial differences in pharmacological as well as toxicological activity (Bruneton, 1995). VB and VC have significant clinical anti-tumor activity [illegible] lymphoblastic leukemia, breast cancer. Based on structure-activity relationships of the naturally occurring dimers, it is evident

that the vindoline-derived moiety of the naturally occurring dimers significantly influences anti-cancer activity of the dimer molecule. For example, it is known that unsaturation at the C6-C7 bond in VB and VC is required for biological activity. If this bond is saturated, anti-cancer activity is significantly eliminated.

As a consequence of the significant clinical anti-tumor activity of VB and VC, much research effort has centered on dimer structure and molecular aspects of alkaloid biogenesis in *Catharanthus*. Metabolic aspects of bisindole biosynthesis in *Catharanthus roseus* have been reviewed by Kutney (1990) and Kutchan (1995). Working with *C. roseus*, Kutney (1990) has summarized the major aspects of dimer synthesis leading to structural differences in the naturally occurring dimers. The bisindole 3',4'-anhydrovinblastine (AHVB), a known naturally-occurring precursor to all of the natural dimers (Kutney, 1990; Endo et al., 1988), also possesses significant anti-cancer activity (IGT pharma, 1998). AHVB differs from VB and VC in that the former possesses structural differences in the catharanthine moiety of the dimer molecule.

Beyond their own inherent chemotherapeutic value, the naturally occurring bisindoles from *Catharanthus* provide useful starting points in the *in vitro* synthesis of structurally related analogs and derivatives. Barnett et al. (1978) prepared deacetylvinblastine amide (vindesine, Figure 2) from VB. Phase I and II clinical trial reports (Dyke and Nelson, 1977) indicate vindesine to be an active oncolytic agent. Clinically, vindesine appears to be less toxic than VC while having an activity spectrum similar to VC rather than its parent VB. The structural similarity of vindesine to VC and VB (the former possessing an amide substitution at C3 on the vindoline moiety) further emphasizes the significance of the vindoline moiety in achieving anti-cancer activity. Conrad et al. (1979), in a comprehensive examination of 41 synthetic N-substituted deacetoxyvinblastine amide sulfates (all synthesized from VB C3 substitutions), further demonstrated the importance of the vindoline moiety in expressing anti-cancer activity; thus, "minor" structural differences in VB modification products attributable to substitutions at the C3 position of the vindoline moiety can be related to the experimental anti-tumor response spectrum and toxicological aspects of the molecule.

Over the course of more than 30 years of research covering structural modification of the natural dimer molecules, various structural synthetic analogs have been produced involving

pharmaceutical properties. European Patent 0,010,458 describes the synthesis of navelbine, a

vinblastine derivative with demonstrated anti-cancer activity. U.S. Patent 5,024,835 describes vinblastine derivatives carrying a detergent chain. U.S. Patent 5,030,620 describes vinblastine-related derivatives containing a protein fragment addition. U.S. Patent 3,352,868 describes the synthesis of dihydrovinblastine by low pressure hydrogenation of vinblastine. The above-mentioned synthetic analogs all possess a single catharanthine and vindoline moiety; all exhibit anti-cancer efficacy though as exemplified by dihydrovinblastine, at levels lower than the natural parent compound. High initial cost of the bisindole reactants (VB - $13, 200/gm; VC - $36,000/gm) coupled with inefficient synthesis and reduced efficacy has resulted in the general failure of synthetic analogs; as well, potency of synthetic derivatives has not surpassed the activity of already-available natural bisindoles (especially vincristine). FDA registered chemotherapeutic *Catharanthus* bisindoles are the naturally produced VB, VC and the synthetic, navelbine.

U.S. Patent 4,199,504 and U.S. Patent 4,203,898 describe bridged bis vinca dimers (i.e., tetramers), wherein the single synthetic molecule consists of two dimer subunits linked at the C3 carbon . Such molecules, and derivatives therefrom, are all active anti-mitotic agents and anti tumor agents. Several of the C3 bridged dimers (e.g. "vinca tetramers") possessed demonstrated activity against transplanted tumors in mice *in vivo*, at dose levels comparable to those used with vincristine and vinblastine. All of the Eli Lilly described tetramers are synthetically derived from naturally-occurring vinblastine or vincristine precursors.

A survey of *Catharanthus* alkaloids, both natural and synthetic, that also show demonstrated anti-cancer activity clearly associates the presence of at least one vindoline moiety with the observed activity; thus, vindoline plays a significant role in the expression of anti-cancer activity. Since monomeric vindoline lacks anti-cancer activity, the catharanthine-to-vindoline carbon-carbon bond is integral to expression of activity. Kutney et al. (1976) describes the nature and specificity of the C(18')-C(15) bond (i.e., C18 of the catharanthine monomer to C15 of the vindoline monomer) as regards to natural configuration and activity expression. Dong et al. (1995) further indicate that there is exquisite sensitivity in the structure activity relationships concerning the stereochemistry at C(18'). The inversion of C(18') configuration from *S* to *R* results in a complete loss of activity. Stereochemistry of other carbons in the dimer molecule are similarly critical concerning the interaction with tubulin (Dong et al., 1995.). Finally, the C(18') ester group,

The long history of *Catharanthus* alkaloid investigations has provided an impetus for study of alkaloids produced by other plant genera. Knowledge of metabolic pathways responsible for *Catharanthus* bisindole intermediates has elucidated general terpenoid biosynthetic schemes leading to dimer alkaloids. Within the Apocynaceae, bisindole alkaloids have been reported from *Tabernaemontana* (van der Heijden et al., 1989), *Stemmadenia* (Valencia et al., 1995), and *Strychnos* (Nuzillard et al., 1996). Based on subfamilial relationships in Apocynaceae (Senbald and Bremer, 1996), and close taxa relationships, based on existence of intergenus somatic hybrids (Kostenyuk et al., 1991), there is likelihood of close structural homology of enzymatic pathways leading to bisindole biosynthesis in these taxa. Stevens et al. (1992) investigated shared enzyme characteristics with respect to alkaloid biosynthesis in *Chinchona*, *Tabernaemontana* and *Catharanthus*. While much of the bisindole pathways are shared in common within Apocynaceae, the absence of vindoline as an alkaloid product in *Tabernaemontana* and other allied genera points to the unique biosynthetic attributes of *Catharanthus*. Vinblastine and vincristine along with other dimers that contain a vindoline moiety, are only known to occur in *Catharanthus*. A principal barrier to melding of metabolic pathways, including those in *Catharanthus,* involves barriers to free intergenus genetic exchange. Overcoming these barriers has, in part, provided impetus to plant transformation involving long-distance transferal of *Catharanthus* genes (Kutchen, 1995; Vasquez-Flota et al., 1997).

Aside from the already discussed catharanthine-vindoline dimers, a single additional dimer, composed of two linked vindoline moieties (vindolicine, Figure 3) has been described by Rabaron et al. (1973). Vindolicine (C_{51} H_{64} N_4 O_{12}, MW=925.086) was isolated from *Catharanthus longifolius*. Rabaron, et al. were the first to recognize the dimeric structure of vindolicine; they also reported the unique UV absorption spectrum of vindolicine. Svoboda et al. (1961) used the name vindolicine to describe a monomeric alkaloid (mol. wt. 457.8) isolated from *Catharanthus roseus*. Based on molecular weight, dimeric vindolicine, as described by Rabaron et al. (1973) has only been isolated from *Catharanthus longifolius*. There are no published reports of anti-cancer activity or known structural derivatives of dimeric vindolicine.

It is desired to identify additional plant alkaloids which will also have biological activity such as anticancer and antifungal activity for mammals and antidisease activity for plants.

SUMMARY OF THE INVENTION

The present invention generally relates to extracts of disease-resistant *Catharanthus* plants, to trimeric and polymeric alkaloids produced by these plants and to the use of the extracts, trimeric alkaloids and polymeric alkaloids as medicinals and anti-biological agents.

More specifically, the present invention relates to the isolation and identification of novel alkaloid compounds produced by complex *Catharanthus* interspecific hybrids. These hybrids contain in part, germplasm of *Catharanthus roseus*, *C. longifolius*, *C. trichophyllus*, *C. scitulus*, *C. pusilus* and other taxa as described by Veyret (1974), or partial combinations thereof. The nature of some of these hybrids has been previously described in U.S. Patent 5,491,285. Biological activity, in the form of *Phytophthora* disease resistance is selected in elite germplasm lines. Disease resistant lines are, in turn, hybridized to enhance biological activity, as detected by increased *Phytophthora* disease resistance. Selected lines, exemplified by enhanced biological activity, are analyzed for alkaloid content using HPLC-MS. Alkaloids are characterized by quantity, quality and correlation with observed disease resistance. Chromatographic peaks are identified by physical data such as UV absorption spectra, retention time, mass spectra, fragmentation patterns and NMR profiles. Peak identities are compared with published physical data and comparisons, where possible, with known standards. Through elimination of known alkaloid constituents, novel trimeric and polymeric alkaloids are isolated and disclosed. These trimeric and polymeric alkaloids, as well as the plant extracts, have biological activity, including anticancer and antifungal activity.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the basic structure of the bisindole alkaloids, vinblastine (R is methyl) and vincristine (R is formyl).

Figure 2 shows the structure of the bisindole alkaloid vindesine.

Figure 3 shows the structure of the bisindole alkaloid vindolicine.

Figure 4 represents an HPLC chromatogram of alkaloids in an extract from *Catharanthus roseus* c.v. "Little Pinkie".

Figure 5 represents an HPLC chromatogram of alkaloids in an extract from *Catharanthus*

Figure 6 represents an HPLC chromatogram of alkaloids in an extract from resistant germplasm, line 18652 (in reference to inventor's line number, such as used in U.S. Patent No. 5,491,825).

Figure 7 represents an ultraviolet absorption spectra for compound 1283, vindolicine and vindoline.

Figure 8 represents an HPLC chromatogram of trimer-containing fraction isolated from sample 18652. Numbers indicate molecular weight of selected peaks.

Figure 9 represents an MS^n fragmentation of compound 1283 indicating principal fragments formed.

Figure 10 represents a first proposed structure for compound 1283.

Figure 11 represents a second proposed structure for compound 1283.

Figure 12 represents a high-resolution HPLC chromatogam of a trimer fraction in the vicinity of retention times 40-46 minutes.

Figure 13 represents UV spectra of selected peaks indicated in Figure 12.

Figure 14 represents an MS^n fragmentation of compound 1351 indicating principal fragments formed.

Figure 15 represents a ^{1}H-NMR spectrum of compound 1351.

Figure 16 represents a COSY plot of compound 1351.

Figure 17 represents an HSQC plot of compound 1351.

Figure 18A, B and C represent a ^{13}C NMR spectrum of compound 1351.

Figure 19 represents a first proposed structure for compound 1351.

Figure 20 represents a second proposed structure for compound 1351.

Figure 21 represents a third proposed structure for compound 1351.

Figure 22 represents a fourth proposed structure for compound 1351.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to extracts of disease-resistant *Catharanthus* plants, to trimeric and polymeric alkaloids produced by these plants and to the use of the extracts, trimeric alkaloids and polymeric alkaloids as medicinals and anti-biological agents.

interspecific hybrids. The novel alkaloid compounds are trimeric or polymeric alkaloids, having

three or more monomeric indole alkaloid moieties. One (or two) of the indole alkaloid moieties is a vindoline and/or catharanthine monomer. On the basis of the data presented herein, it is believed that an additional monomer(s) in the novel alkaloid compounds of the present invention is a vindoline-based moiety. In one preferred embodiment, the novel alkaloid compound is a trimer containing three vindoline or vindoline-based monomers. The novel alkaloids of the present invention have molecular weights in the range of about 980 to about 2000 daltons. The alkaloids as isolated from the plant tissues may be saturated or unsaturated with respect to the C6-C7 bond in the vindoline monomer(s). It is preferred that the alkaloids be unsaturated, since it is the unsaturated form which has the highest biological activity. The novel alkaloids of the present invention are isolated from extracts of disease-resistant *Catharanthus* plants.

DEFINITIONS

The present invention employs the following definitions:

"**Alkaloid**" refers to a cyclic organic compound containing nitrogen in a negative oxidation state and which is defined by Bruneton to be of limited distribution among living organisms. (Bruneton, 1995).

"**Catharanthine**" refers to the chemical as described in Chapman and Hall (1997), including racemic and isomeric mixtures thereof.

"**Indole alkaloid**" refers to an alkaloid compound which arises from strictosidine and which possess an indole ring structure. (Bruneton, 1995).

"**Monomer**" or "**subunit**" refer to an alkaloid having a molecular weight of less than 458 daltons, and includes those structures described in Lounasmaa and Galambous (1989).

"**Trimer**" refers to a single chemical entity composed of three indole monomers with a molecular weight of greater than 980 daltons.

"**Vindoline**" refers to the chemical as described in Chapman and Hall (1997), including racemic and isomeric mixtures thereof.

"**Vindoline-based**" refers to a compound which is wholly or in part derived from vindoline or its immediate precursers.

Chromatographic peaks are identified by physical data such as UV absorption spectra, retention

time, mass spectra, fragmentation patterns and NMR profiles. Peak identities are compared with published physical data and comparisons, where possible, with known standards. Through elimination of known alkaloid constituents, novel trimeric and polymeric alkaloids are isolated by HPLC fractionation.

More specifically, greenhouse grown and field grown *Catharanthus* plants, selected for enhanced biological activity against *Phytophthora* disease resistance, are assayed for alkaloid content using common methods known to those skilled in the art and as described in the examples below. Any suitable extraction method can be employed to prepare the *Catharanthus* extract. Suitable isolation and separation methods for indole alkaloids have been reviewed by Svoboda (1964), Verpoorte (1987) and U.S. Patent 4,172,077. According to one embodiment of the present invention, mature leaves of the selected *Catharanthus* plants are collected and frozen. The frozen leaves are fractured and ground to a coarse powder and extracted in methanol. The methanol is evaporated and the gum is acid extracted. Any suitable acid can be used for the acid extraction. However, it is preferred to use weak organic acids, such as tartaric or citric acid, to maximize the isolation of unsaturated alkaloids. The acid extracts are basified and the aqueous phase is extracted with an organic solvent. The organic solvent is evaporated to produce the alkaloid sample. The alkaloid sample is redissolved in methanol and subjected to HPLC to further fractionate the extracts. Alternatively, crude alkaloid extracts are subjected to size exclusion ambient pressure column chromatography and HPLC to isolate individual alkaloid compounds. The alkaloid compounds are identified on the basis of their molecular weight, including alkaloids having molecular weights in the range from about 980 to about 2000 daltons. Other physical characteristics are shown for several of the alkaloids of the present invention.

Extracted samples are analyzed by high pressure liquid chromatography (HPLC), and photo diode array detection (PDA). Analysis methods are discussed by McCloud et al. (1997). Where appropriate, mass spectrometry is used to further clarify molecular structural aspects of detected alkaloids, as used by Verpoorte and Niessen (1994) and Chu et al. (1997). Nuclear magnetic resonance (NMR) is also employed to characterize structures of indole alkaloids using methods known to those in the art and exemplified by Mukherjee et al. (1997) and Andre-Touche et al. (1997). On the basis of the evidence gathered at this time, several possible structures exist

The novel trimeric and polymeric indole alkaloids of the present invention are produced in plants. These naturally-occurring products reflect metabolic activity in stereospecific pathways. HPLC-MS of trimer containing fractions revealed multiple discreet chromatographic peaks for several alkaloids, such as those having m/z 1231.3 and 1241.5. The presence of distinct chromatographic peaks with different retention times yet also with identical mass and UV absorption spectra indicates presence of stereospecific isomeric forms differing in configurational and conformational arrangement. Exquisite sensitivity in structure-activity relationships described for known bisindoles (including VB, VC) suggests that stereospecificity in trisindoles may also be significant in determining bioactivity. Detection of trimeric stereoisomers suggests that presence of multiple forms likely influences observed bioactivity of the trimers.

Yield of trimers is influenced by both genetic and environmental factors. Figure 6 illustrates attainable yield of compound 1283, relative to VB and other associated monomers and dimers. Considering that VB is the starting material from which various medicinal alkaloids are synthesized, the yield of compound 1283 (compare Figures 4 and 6) significantly exceeds that of VB in potential commercial production germplasm approximated by Figure 4.

Attempts to increase alkaloid yield in *Catharanthus roseus* via biotic or physical elicitation have been reported for suspension cell cultures (Godoy-Hernandez and Loyola-Vargas, 1996; Moreno et al., 1996). While increased yield of some monomers (ajmalicine, serpentine) has been reported (Shanks et al., 1998), increased yield of dimers remains unachieved especially in whole plants, despite thirty or more years of intense effort.

Yield of trimers comprising the instant invention of this patent is elicitable. Through manipulation of appropriate biological and physical factors, trimer yields can be increased from ambient levels where the amount of compound 1283 approximates that of VB, to that shown in Figure 6 where the amount of compound 1283 is 13 times greater than VB. Thus, unlike dimers, the production of trimers/polymers can be elicited . Factors responsible for elicitation of trimers include manipulation of the whole-plant pH environment and provision with sulfate. Though not in *Catharanthus*, Sikuli and Demeyer (1997), reported increased hyoscyamine yield in *Datura stramonium* in culture medium in which SO_4^{2-} and K^+ were dominant. Elicitors such as acetylsalicylic acid and salicylic acid do not appear to influence trimer yield.

(Figure 9), compound 1351 (Figure 13), and other trimers yielded fragments structurally similar

to known dimeric and monomeric indole alkaloids. Based on molecular weight, fractionation of 1283, 1351, VB and VC all yield fragments in common indicating, as expected, close structural homology. Since trimers contain a second vindoline moiety when compared to VB or VC, controlled *in vitro* degradation resulting in the loss of a single vindoline moiety, readily yields VB, VC, or related bisindoles. In addition to synthesis of VB or VC from trimers, the trimers or trimer derivatives, themselves, are used as stereospecific starting materials in the synthesis of novel bisindoles with bioactivity paralleling the activity of known VB and VC derivatives. In addition, the trimeric and polymeric alkaloids of the present invention are used as starting materials for the preparation of bioactive derivatives as described, for example, by Barnett (1978), Conrad et al. (1979), European Patent No. 0,010,458 and U.S. Patent Nos. 5,620,985, 5,024,835, 5,030,620 and 3,352,868.

Disease-resistant plants of U.S. Patent 5,491,285 uniquely produce trimeric and polymeric indole alkaloids. These same plants exhibit profound antibiological activity in the form of elevated disease and pest resistance. Production of dimeric alkaloids, for example VB and VC (see Figure 4), affords mild disease resistance in comparison to vindoline-lacking mutants as shown herein. Thus, the functional role of dimer/trimer/polymer alkaloids *in planta* can be attributed to antibiological defensive action. When extracted, purified and administered to humans, this same antibiological defensive activity of dimers constitutes the long-established role of medicinal *Catharanthus* bisindoles, especially VB and VC. The striking structural similarity of trimers and polymers to bisindoles, coupled with the observed profoundly heightened antibiological expression characterized by trimer/polymer containing plants clearly reflects the potency of trisindole bioactivity. Demonstrated antifungal activity and suppression of predation by diverse pests including mites, insects, and molluscs indicate use of trisindoles and polyindoles in both animals and plants. The usefulness of the trimeric and polymeric alkaloids as antifungal and anticancer agents is demonstrated by antifungal screening assays and anticancer screening assays such as described herein.

The present invention encompasses the use of the novel trimeric and polymeric alkaloids in pharmaceutical and therapeutic modalities for anticancer or antifungal activities. The alkaloids of the present invention can be formulated in pharmaceutical compositions, which are prepared

pharmaceutically acceptable salts of the active agent. These compositions may comprise, in

addition to one of the active substances, a pharmaceutically acceptable excipient, carrier, buffer, stabilizer or other materials well known in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The carrier may take a wide variety of forms depending on the form of preparation desired for administration, e.g., intravenous, oral, intrathecal, epineural or parenteral, and depending on the therapeutic modality. The compounds are administered in similar manner as other biologically active plant alkaloids, such as vincristine and vinblastine, or they are administered in a similar manner as other antifungal agents.

For oral administration, the compounds can be formulated into solid or liquid preparations such as capsules, pills, tablets, lozenges, melts, powders, suspensions or emulsions. In preparing the compositions in oral dosage form, any of the usual pharmaceutical media may be employed, such as, for example, water, glycols, oils, alcohols, flavoring agents, preservatives, coloring agents, suspending agents, and the like in the case of oral liquid preparations (such as, for example, suspensions, elixirs and solutions); or carriers such as starches, sugars, diluents, granulating agents, lubricants, binders, disintegrating agents and the like in the case of oral solid preparations (such as, for example, powders, capsules and tablets). Because of their ease in administration, tablets and capsules represent the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are obviously employed. If desired, tablets may be sugar-coated or enteric-coated by standard techniques. The active agent can be encapsulated to make it stable to passage through the gastrointestinal tract while at the same time allowing for passage across the blood brain barrier. See for example, WO 96/11698.

For parenteral administration, the compound may be dissolved in a pharmaceutical carrier and administered as either a solution or a suspension. Illustrative of suitable carriers are water, saline, dextrose solutions, fructose solutions, ethanol, or oils of animal, vegetative or synthetic origin. The carrier may also contain other ingredients, for example, preservatives, suspending agents, solubilizing agents, buffers and the like. When the compounds are being administered intrathecally, they may also be dissolved in cerebrospinal fluid.

For topical administration, the active agent is added to a carrier useful for topical administration. The carrier can vary widely depending on the site for topical administration. Formulations suitable for topical administration to the skin may be presented as ointments, creams,

suitable for vaginal administration may be presented as tampons, creams, gels, pastes, foams or spray formulations containing, in addition to the active agent, suitable carriers.

The active agent of the present invention, when used as an anticancer agent, is administered in the same manner as vinblastine or vincristine. Because of severe toxic reactions, vinblastine and vincristine are administered by an IV drip.

The active agent is preferably administered in a therapeutically effective amount. The actual amount administered, and the rate and time-course of administration, will depend on the nature and severity of the condition being treated. Prescription of treatment, e.g. decisions on dosage, timing, etc., is within the responsibility of general practitioners or specialists, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of techniques and protocols can be found in *Remington's Pharmaceutical Sciences*.

Alternatively, targeting therapies may be used to deliver the active agent more specifically to certain types of cell, by the use of targeting systems such as antibodies or cell specific ligands. Targeting may be desirable for a variety of reasons, e.g. if the agent is unacceptably toxic, or if it would otherwise require too high a dosage, or if it would not otherwise be able to enter the target cells.

Catharanthus seeds resistant to *Phytophthora* have been placed on deposit with the American Type Culture Collection, Manassas, Virginia, under Deposit Accession Number 75636 on 14 January 1994 in connection with U.S. Patent No. 5,491,285. All restrictions on the availability of the seed have been lifted in connection with said patent.

EXAMPLES

The present invention is described by reference to the following Examples, which are offered by way of illustration and is not intended to limit the invention in any manner. Standard techniques well known in the art or the techniques specifically described below were utilized.

EXAMPLE 1

Extraction of *Catharanthus roseus* alkaloids

frozen leaves were fractured and ground to a coarse powder and forced-air dried at 10 °C for ca.

24 hrs. The dried leaves were extracted in methanol (ca. 1 hr.) and filtered. The filtrate was evaporated to yield a dark gum. The gum was twice extracted for ca. 1 hour with 1% H_2SO_4, the extracts combined, filtered and adjusted to pH 9 by addition of ammonium hydroxide or other suitable base. The aqueous phase was extracted three times with ethyl acetate, methylene chloride or other appropriate organic solvent (see Verpoorte, 1987). The organic extracts were combined, filtered, dried where appropriate over anhydrous sodium sulfate or other suitable agent, and evaporated, yielding the alkaloid sample.

The alkaloid sample was redissolved in a small amount of methanol and analyzed with HPLC and PDA detection utilizing standard methods known to those in the art (see Shanks et al., 1998). Analysis of extracts (Figure 4) revealed the presence of several expected monomeric alkaloids including but not limited to strictosidine, catharanthine and vindoline. Dimeric indole alkaloids were also detected including, but not limited to vinblastine, vincristine, leurosine, leurosidine, 3', 4'- anhydrovinblastine and catharine. PDA detection allowed for quantification and peak purity checking. Peak identities were confirmed by retention times and ultraviolet absorption spectra. Peak identities were validated by comparison with standards of strictosidine, catharanthine, vindoline, vinleurosine, lochnerine, vindolicine, catharine, vinblastine, and vincristine, as well as physical data from the literature, including Sangster and Stuart (1965) and Neuss (1963). Peak areas in Figure 4 indicate relative abundance and composition of selected chromatographic peaks in *Catharanthus roseus* cv. "Little Pinkie."

EXAMPLE 2

Extraction of *Catharanthus longifolius*

Leaves of *Catharanthus longifolius* (Pichon) were prepared and extracted as in the previous example. HPLC analysis revealed the presence of several monomeric and dimeric alkaloids in common with *Catharanthus roseus* (Figure 5). The alkaloid chemistry of minor *Catharanthus* spp. is also reported by Tin-Wa and Farnsworth (1975). In addition to the dimeric alkaloids found in *Catharanthus roseus*, another bisindole, vindolicine (Figure 3) was detected in *C. longifolius*. Rabaron et al. (1973) also found vindolicine in *C. longifolius* and further structurally characterized the dimer molecule as being composed of two vindoline subunits linked

physical characteristics

confirmed in the present extracts. To date dimeric vindolicine has only been reported in C.

longifolius. Svoboda et al. (1961) identified a monomeric alkaloid having a molecular weight of 457.8 as vindolicine which should not be confused with the dimeric compound of Rabaron et al. (1973), and whose structure is confirmed in Chapman and Hall (1997).

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EXAMPLE 3

Interspecific Crosses In *Catharanthus* as Source of Germplasm for Alkaloid Isolation

Germplasm of *Catharanthus* species was grown to flowering maturity in a greenhouse using methods known to those skilled in the trade. Species included in a complex crossing program are listed in Table 1. Confirmation of hybridity was established by tracking morphological, reproductive and phytochemical traits, including *Phytophthora* disease resistance (see U.S. Patent No. 5,491,285). More than 9,000 hybrid lines, and selections therefrom provided genetic backgrounds which could be assessed for alkaloid content and inheritance patterns. Extreme variance in morphological and reproductive forms suggested that a parallel variability in phytochemical biotypes might also be present.

TABLE 1

Species and Interspecies Hybrids Investigated for Alkaloid Content.

Catharanthus roseus
C. longifolius
C. trichophyllus
C. scitulus
C. pusilus
C. roseus biotypes[1]
C. roseus cultivars[2]
C. roseus x *longifolius*[3]
C. roseus x *trichophyllus*[3]
C. roseus x *scitulus*[3]
C. roseus x *roseus* biotypes[3]
C. roseus x *longifolius* x *trichophyllus* x *scitulus* x *roseus* biotypes x *roseus* cultivars [3,4]

[1] Non-typical forms of *C. roseus* see U.S. Patent No. 5,491,285. Includes *Phytophthora* resistance gene of U.S. Patent No. 5,491,285.
[2] Including but not limited to cultivars in U.S. Patent No. 5,491,285, Table 1.
[3] Includes cross and reciprocal, backcrosses, and multiple succeeding generations including backcrosses.
[4] Includes in excess of 12 generations and more than 9,000 breeding crosses.

EXAMPLE 4

As indicated in Table 1, germplasm of the *Phytophthora* resistance gene described in U.S. Patent 5,491,285, was included in the crossing program. Extraction of alkaloids from this

resistance stock utilized methods of Example 1. A chromatogram of alkaloids derived from a line containing the resistance gene is shown in Figure 6. PDA evaluation of chromatographic peaks revealed the monomeric and dimeric alkaloids characteristic of *Catharanthus roseus*. As well, resistance germplasm also produces small amounts of vindolicine, as indicated by the peak shown in Figure 6. Detailed examination of other chromatographic peaks indicated their presence in the resistance line, *C. roseus*, *C. longifolius*, or combinations thereof. A chromatographic peak at an approximate retention time of 43.3 minutes (Figure 6) was characteristic of lines containing the resistance gene; this same peak was not present in *C. roseus* of Example 1 or *C. longifolius* of Example 2. Exhaustive attempts to detect this peak using concentrations from large amounts of plant material and other methods known to those skilled in the art of HPLC, failed to reveal the presence of the peak with retention time 43.3 min. in any examined germplasm of *C. roseus* or *C. longifolius*. Examination of numerous lines containing the resistance gene of U.S. Patent 5,491,285 revealed a perfect correlation with presence of the resistance gene; conversely, lines segregating for sensitivity (non-resistant) lacked detectable signal for the chromatographic peak at approximately 43.3 minutes.

Absolute amounts of the novel peak, as indicated by relative comparisons of peak areas of known dimers, especially vinblastine, indicated total amounts of the novel compound were variable, dependent on the resistant line examined.

EXAMPLE 5

Extraction of Commercial *Catharanthus* Lines

Commercially available *Catharanthus* germplasm, including but not limited to those cultivars listed in Table 1 of U.S. Patent No. 5,491,285 were examined for presence of the novel peak described in Example 4. All obtainable cultivar germplasm failed to contain the novel compound of Example 4, using the highest attainable resolution capable with PDA instrumentation (Model 996, Waters Corporation, Milford, MA). All available *Catharanthus* germplasm lines available from the United States Dept. Agriculture (GRIN, Beltsville, MD) similarly failed to possess the novel compound. To date, the novel compound as shown by the chromatographic peak at an approximate retention time of 43.3 minutes (Figure 6) and as further characterized herein has

EXAMPLE 6

Peak Purity

As indicated, all resistant lines expressing *Phytophthora* disease resistance, characterized by the methods expounded in U.S. Patent No. 5,491,285, possess finite quantities of the novel alkaloid described in Example 4. In order to better exemplify the physical characteristics of this novel compound, selected genetic lines such as line 18652 were grown in the greenhouse and field to produce larger amounts of the compound in question. Larger samples were HPLC injected and the eluate corresponding to the 43.3 minute peak was collected, using common HPLC methodology known to those in the art. The peak was re-extracted at low pH, a portion again subjected to HPLC analysis and analyzed for peak purity utilizing common peak analysis protocols (Millennium ver. 2.15, Waters, Milford, MA). Results indicated a single compound with a ultraviolet absorption as indicated in Figure 7, which compares the UV absorption spectra of compound 1283 with vindolicine and vindoline. HPLC analysis of additional portions at high pH (7.5) further confirmed the presence of a single pure compound, characterized by a slight bathochromatic shift, a feature common to indole alkaloids (Sangster and Stewart, 1965). Comparison of the UV spectrum (Figure 7) of the novel compound with existing spectra libraries (Sangster and Stewart, 1965; Neuss, 1963) failed to reveal a match, further indicating novelty; the spectrum is, however, reasonably close to that of vindoline (Neuss, 1963) and vindolicine (Rabaron et al., 1973) suggesting close structural affinity.

EXAMPLE 7

Mass Spectroscopy: Molecular Weight

Portions of the pure compound obtained in Example 6 were analyzed with mass spectroscopy (Finnigan, San Jose, CA). Results confirmed a single-charged pure compound of m/z 1283.5 for the novel compound. Analyses of multiple samples derived from different germplasm lines resistant to *Phytophthora* including but not limited to 18652 and 18795 and isolated with variant extraction methodologies, such as disclosed in Svoboda (1964), Verpoorte (1987) and U.S. Patent No. 4,172,077, repeatedly yielded the same compound. It was further found that any method suitable for extracting indole alkaloids from plant tissue repeatedly yielded

Table 2 indicates known molecular weights for alkaloids isolated from *Catharanthus*, allied Apocynaceae genera and other organisms. Notably, the highest molecular weight for any known alkaloid isolated from any *Catharanthus* species is 925 (vindolicine, Chapman and Hall, 1997). Vindolicine also possesses the largest molecular weight reported for any indole alkaloid. Stepwise increase in molecular weights (Table 2) suggested the novel compound might be a polymeric alkaloid composed of multiple monomeric units. Since all known *Catharanthus* dimers are composed of catharanthine and/or vindoline moieties, possible molecular weights were calculated for trimers based on combinations of catharanthine and vindoline moieties derived from both vinblastine and vincristine (Table 3). The extreme similarity of the observed m/z (1283.5) and the calculated molecular weight of trimers containing a single catharanthine and two vindoline moieties strongly implicated the novel compound as a trimer indole alkaloid.

TABLE 2

Molecular Weights[1] of Naturally-Occurring Alkaloids

Alkaloid	Source	Family	Class[2]	Molecular Wt.
vindoline	*Catharanthus*	Apocynaceae	M	456
catharanthine	*Catharanthus*	Apocynaceae	M	336
vincristine	*Catharanthus*	Apocynaceae	D	825
vinblastine	*Catharanthus*	Apocynaceae	D	811
leurosine	*Catharanthus*	Apocynaceae	D	809
catharine	*Catharanthus*	Apocynaceae	D	823
vinamidine	*Catharanthus*	Apocynaceae	D	825
3, 4 -anhydro-vinblastine	*Catharanthus*	Apocynaceae	D	793
vindolicine	*Catharanthus*	Apocynaceae	D	924[3]
conoduramine	*Tabernaemontana*	Apocynaceae	D	703
voacamine	*Tabernaemontana*	Apocynaceae	D	703
tabernaelegantinine	*Tabernaemontana*	Apocynaceae	D	762[4]
michellamine B	*Ancistrocladus*	Ancistrocladaceae	D	770
Hamacanthin A	*Hamacantha*	(marine sponge)	D	486
Panganensine	*Strychnos*	Loganiaceae	D	587

[1] Reported as molecular weight of free base.

[2] Monomer (M) or dimer (D).

[3] Highest molecular weight reported for any naturally-occurring indole alkaloid.

TABLE 3

Theoretical Molecular Weight of Trimers[1].

Trimer Combination	Expected Molecular Weight
VB+ V(VB)	1265.5
VB+ V(VC)	1279.5
VC+ V(VB)	1279.5
VD+ C(VB)	1279.5
VC+ V(VC)	1293.5
VD+ V(VB)	1379.6
VD+ V(VC)	1393.6
V(VC)+ V(VC)+V(VB)	1393.5
V(VC)+ V(VC)+V(VC)	1407.5
VB+ VC	1633.9
VB+ VD	1734.0
VC+ VD	1748.0

[1] abbreviations: VB =vinblastine, VC = vincristine, VD= vindolicine, V(VB) = vindoline moiety as in vinblastine or vindolicine, V(VC) = vindoline moiety as in vincristine, C(VB) = catharanthine moiety as in VB or VC.

EXAMPLE 8

Liquid Chromatography: Trimer Fractions

Endo et al. (1987) used Sephadex LH-20 to separate *Catharanthus roseus* dimeric alkaloids from monomers. Crude whole alkaloid extracts obtained as described in Example 4 were subjected to size exclusion ambient pressure column chromatography using Sephadex LH-20. As is known to those in the art, separations are accomplished based on molecular size, which in turn, is dependent on molecular weight. Sequential column chromatography fractions were collected and analyzed by HPLC as described in Example 4. As expected, the earliest size-exclusion fractions were devoid of all dimeric alkaloids (vinblastine, etc.) but did contain the heavier novel trimeric alkaloid (m/z 1283) at its expected retention time. Moreover, additional peaks were detected in the 1283-containing fraction, indicating presence of previously undetected trimers or polymers. In non-fractional analyses (as in Figure 6) these additional trimer peaks had been hidden by co-eluting monomers of substantially greater abundance. Combined UV spectral data and retention times confirmed the identity of compound 1283 and differentiated the other trimer/polymer compounds. Increased quantities of trimers were obtained by large-scale column

EXAMPLE 9

HPLC-MS

Trimer-rich fractions obtained as described in Example 8 were subjected to further analysis by combined HPLC-mass spectroscopy (HPLC-MS). Peaks eluting from the HPLC column are directly shunted to mass detection, thereby correlating retention time, peak purity, and UV absorption spectra with molecular weight and fractionation patterns. Figure 8 shows the HPLC chromatogram of a trimer fraction with corresponding selected molecular weights (m/z) as indicated. Significant peaks having molecular weights of 982. 1163, 1231, 1241, 1281, 1283 and 1351 are shown in this Figure. A complete analysis of the chromatogram showed that discreet alkaloids with m/z 982, 1127, 1145, 1154, 1163, 1182, 1193, 1231, 1241, 1247, 1253, 1263, 1269, 1279, 1281, 1283, 1299, 1305, 1325, 1351, 1352, 1422, 1453, 1456, 1533, 1535, 1653, 1738, 1747, 1766, 1870, 1958, and 1973 were also detected. These alkaloids were further characterized with additional physical data, including mass fragmentation patterns, confirming their identity as indole alkaloids related to VB and VC. All of these peaks exceed the highest known molecular weight for any reported indole alkaloid (vindolicine, Table 2, mol. wt. 924). Therefore, the compounds of these peaks comprise a novel class of trimer/polymer alkaloids constructed, at least in part, from monomeric catharanthine and vindoline entities. As is known to those in the art, baseline perturbations likely reflect additional trimer/polymer alkaloids whose HPLC-MS signals were below resolution levels of the instrumentation employed. Thus, additional trimer peaks were detected and quantified although they were not fully characterized.

EXAMPLE 10

Analysis of Extracts for Artifacts or Degradation

As with other organic constituents, alkaloids are subject to degradation and autolysis, dependent on extraction, storage and analysis methods employed. Verpoorte (1987) has suggested that halogenated solvents can induce artifacts in indole alkaloids. Trimer containing fresh leaves were subjected to repeated extractions using diverse solvents, acids, bases, solid phase extraction and other methods known to those in the art, including those described in Svoboda (1964), Verpoorte (1987) and U.S. Patent No. 4,172,077. Results from these, as well as other known

natural products rather than artifacts induced by specific analysis protocols.

Sethi and Thimmaiah (1985) and Thimmaiah and Sethi (1985) reported on degradation of vinblastine and vincristine, induced by both biotic and abiotic factors. In all instances, degradation products were characterized by lower molecular weights than the parent compounds. *Catharanthus* bisindoles are also light and heat labile (U.S. Patent No. 4,831,133; Bommer et al., 1964). Trimer containing leaf samples were intentionally subjected to adverse drying conditions, extracted and subsequently analyzed for dimer and trimer alkaloid content. Loss of trimer peaks was concomitant with loss of dimer peaks, further substantiating that trimer compounds are natural products which are chemically reactive in manners similar to other indole alkaloids. Induced degradation of bisindole standards and trimer extracts similarly results in loss of higher molecular weight components, further substantiating that trisindoles are naturally synthesized *in planta* products.

EXAMPLE 11

C6-C7 Saturation of Vindoline Moieties

Catharanthus bisindoles, including vinblastine, vincristine, vindesine and derivatives therefrom, are known to undergo reduction of the 6,7 double bond in the vindoline moiety, resulting in a +2 change in molecular weight (see Figure 1). Noble et al. (1967) described the resulting increase of molecular weight (+2) in dihydrovinblastine compared to the unsaturated vinblastine. Dihydrovinblastine can be produced by hydrogenation of vinblastine under acetic conditions(U.S. Patent No. 3,352,868). Bieman (1964) demonstrated a +2 increase in molecular weight for other dihydro-bisindole alkaloids containing a vindoline moiety. Reduction of the 6,7 double bond in vinblastine (U.S. Patent No. 3,352,868) results in a substantial (13x) loss of anti-cancer activity. Reduction of the 6,7 double bond in vindesine similarly results in substantial reduced anti-cancer activity (Barnett et al., 1978).

High resolution mass spectroscopy of the 1283 compound revealed a m/z of 1283.5850. As indicated in Table 3, trimers composed of a single catharanthine and two unsaturated vindoline moieties would be expected to have a molecular weight of 1279.5. Saturation of the 6,7 double bond of both vindoline moieties would yield the observed molecular weight of 1283.5. As explained in Example 1, trimer containing leaves were extracted in a strong mineral acid (H_2SO_4),

presence of two small chromatographic peaks immediately preceding elution of the 1283 peak.

The previously unanalyzed peaks had m/z of 1279.5 and 1281.5, respectively. When additional trimer containing leaf samples were extracted with tartaric acid, a weak organic acid (Svoboda, 1964), instead of sulfuric acid, the abundances of the 1279.5 and 1281.5 peaks increased relative to the area of the 1283.5 peak. Thus, naturally occurring trisindoles with m/z 1279.5, 1281.5 and 1283.5 were extracted and isolated when tartaric acid was used in place of sulfuric acid. Similar results are obtained when citric acid, another weak organic acid, is used in place of sulfuric acid in the extraction of trimer containing leaves. The respective relative yields were dependent upon extraction parameters and methodologies employed. Detailed analysis of other trimers, as isolated in Example 9, indicated the presence of unsaturated and saturated forms, dependent on the vindoline moieties in the compound. Similarly with the results achieved using tartaric acid or citric acid for extraction with respect to compound 1283, unsaturated trimers are isolated with respect to the saturated trimers identified in Example 9.

Barnett et al. (1978) reported the formation of vindesine N_b-oxides upon prolonged storage of vindesine free base. The N_b-oxide, characterized by a net increase of m/z +16 (corresponding to an added oxygen) could be reduced back to the free base form. HPLC-MS analysis of a trimer enriched fraction showed that the primary constituent comprised compound 1283 and that two additional peaks, both with m/z 1299 eluted earlier. These earlier eluting peaks are consistent with a lower pK_a expected of the 1283 N_b-oxides. That two distinct peaks with +16 m/z were found reflects isomeric forms of the N_b-oxides, chromatographically discernable because of slight differences in pK_a. Existence of 1283 N_b-oxides (m/z 1299) further verifies the existence of vindoline moieties in the trimer compounds.

EXAMPLE 12

Characterization of Compound 1283

A detailed comparison of UV absorption spectra for compound 1283, vindoline, and vindolicine is provided in Figure 7; all samples were analyzed under identical solvent and pH conditions. Spectral analysis reveals close similarity between the three compounds. Mass spectral analysis (MS^n, Finnigan, San Jose, CA) of an infused sample of compound 1283 revealed the fragmentation indicated in Figure 9. Fragmentation patterns for other trimers and standards of VB

Figure 10. As known to those in the art of chemical structure determinations, other structural

arrangements may also be present other than that indicated in Figure 10, including the proposed structure shown in Figure 11, that can explain the observed physical characteristics and bioactivity of compound 1283.

High resolution HPLC of a trimer containing fraction extracted from line number 18733 is shown in Figure 12. Selected peaks in near vicinity to compound 1283 (retention time 42.386 in this trace) are integrated as shown. Corresponding UV absorption spectra for integrated peaks of Figure 12 are shown in Figure 13 (marked by retention time). Discreet chromatographic peaks with such close retention times and similar absorption spectra suggest multiple detected isomeric or racemic forms. For example, peaks with retention times 42.38 (compound 1283) and 43.55 possess essentially identical UV spectra. These molecular forms likely vary structurally by bond angle or substitution position but may not possess differing molecular weights. Detection of distinct chromatographic peaks demonstrates that trimer metabolic pathways generate numerous molecular forms in addition to those indicated in Figures 10-11. These closely related structural forms vary by relative abundance as indicated in Figure 12. Collectively, Figures 10-13 indicate presence of multiple molecular forms generally represented in Figures 10-11, though not having exactly the same trimeric structural configuration as illustrated.

EXAMPLE 13

Characterization of Compound 1351

Using methods as described in Example 12, compound 1351 was further characterized.. The ultraviolet absorption spectrum is characterized as Λ_{max} 215.9, 245.2, 314.9. Principal MS ^{n} fragmentation products of compound 1351 are shown in Figure 14.

A composite sample of comound 1351 was obtained by combining multiple HPLC fractions containing the 1351 peak. An approximate 3 mg sample of compound 1351 was analyzed by NMR to yield ^{1}H NMR, COSY, HSQC, ^{13}CNMR, and MS data as described below.

1H NMR (Figure 15):

Key pieces of structural information derived from the ^{1}H NMR spectrum are listed below:

1. There are five equally integrated aromatic protons (~6.2-7.6 ppm). Because all are indicated as singlets, the trimer may not contain a catharanthine moiety, and instead

[illegible]

triplets). This supports the trimer structure based on three vindoline based subunits.

3. There are five methyl groups attached to oxygen as either methoxy moieties or esters (~3.6 ppm singlets).
4. There are three methyl groups attached directly to a carbonyl carbon (~2.8 ppm singlets).
5. There are six olefinic protons:

--3 as a ~5.9 ppm multiplet, corresponding to the vindoline carbon position 7 in all three subunits (see Figure 1 for numbering convention in the vindoline moiety).

--3 as doublets from 5.5-5.7 ppm, corresponding to position 6 in all three subunits.

6. There are three methyl groups attached to nitrogen (~1.99-2.05 ppm singlets).

COSY (Figure 16):

The COSY data plots ^{1}H NMR spectra on both X and Y axes. Correlations between protons indicate that they are attached to adjacent carbons. COSY information is summarized below:

1. The aromatic signals showed no correlations. This was expected because they were singlets in the ^{1}H NMR spectrum and so should have no protons attached to adjacent carbons.
2. The molecule is complex in the aliphatic region.
3. One of the methyl groups of the ethyl side chains is in a different chemical environment from the other two. The protons at 0.6 ppm show correlations to protons resonating at ~1 ppm and at 1.75 ppm. The protons at 0.8 ppm show correlations to protons resonating at ~1 ppm and 1.90 ppm. However, the protons at 0.7 ppm show correlations to protons resonating at 1.6 and 1.85 ppm. This indicates that there is something different near the ethyl side chain between this monomer and the other two. While this difference could be due to confirmational variance, it could also indicate some different functionality in the vicinity of this side chain.

HSQC (Figure 17):

HSQC is a two dimensional experiment that correlates the peaks of a ^{1}H NMR spectrum

1. 35 proton bearing carbons are detected (non-protonated carbons do not appear in HSQC).
2. The C 17 position has a unique chemical shift (~95 ppm) in the ^{13} C spectrum. The HSQC shows only two correlations from aromatic protons (6.2 and 6.3 ppm) to carbons resonating at ~95 ppm, not three. Since the molecule is a trimer, this implies that one of the monomer units is not protonated at the C 17 position, while the other two are. It is also possible that an alcohol is attached at this C 17 position and the connection to the other monomer units is at the C 3 position, as in other parts of the trimer molecule.
3. Observed correlations also support the presence of the ethyl side chains in the molecule.

^{13}C NMR (Figure 18):

Despite the undesired signal to noise ratio of Figure 18, the NMR software was able to evaluate 55 of the 74 detected signals. Table 4 summarizes salient aspects derived from the ^{13}C NMR.

TABLE 4

Summarized ^{13}C NMR Information

number of signals expected	type of carbon responsible for this signal	expected chemical shift	observed
6	carbonyl carbons	~170-172 ppm	5 signals in this region
4	Ar-O-R (aromatic carbons with oxygens attached to them, ie. Ar-OMe or Ar-OH)	~150-160 ppm	5 signals in this region
3	position 18 carbons	~150 ppm	
3	methyls that are part of the ethyl side-chain	~7-9 ppm	3 signals in this region

MS:

analyzed by HPLC and MS. A degredation product with m/z 457 (vindoline) was readily

detected; thus; a single vindoline unit can be generated from degredation of compound 1351. Another degredation product with m/z 937, corresponding to vindoline+vindoline+acetate, was readily detected. Thus, upon exposure to adverse conditions, the 1351 compound can degrade to release either one separate or two adjacently linked vindoline moieties.

Combined physical data indicate a possible structure for compound 1351 as shown in Figure 19. As known to those in the art of chemical structure determinations, other structural arrangements may also be present beyond that indicated in Figure 19, including the proposed structures shown in Figures 20-22. Just as with compound 1283, high resolution HPLC of trimer enriched fractions revealed multiple though small discreet chromatographic peaks near the primary 1351 peak of Figure 8. Several of these trace peaks possess UV spectra substantially similar to that of compound 1351. Thus, though not of equal relative abundance, multiple isomeric or racemic forms of compound may coexist encompassing at least those structures indicated in Figures 19-22.

EXAMPLE 14

Characterization of Additional Trimers and Polymers

Additional trimers and polymers as indicated, in part in Figure 8 and Example 9, are described in Table 5. Extraction parameters, specific for indole alkaloids, yielded these compounds which were additionally characterized by the indicated physical data. Combined evidence indicates that these compounds are indole alkaloids. Based on similarity to calculated molecular weights for multiple monomeric units (Table 3), the compounds of Table 5 are trimers or polymers composed of multiple monomeric units, especially catharanthine and vindoline.

TABLE 5

Characteristics of Trimers and Polymers

Molecular weight (m/z)	UV absorption (Λ_{max})
982	213.6, 260.5, 309.0
1127	
1165	213.6, 249.9, 309.0
1182	

1193	214.8, 258.2, 306.6
1231	214.8, 246.4, 316.1
1241	213.6, 249.8, 309.0
1247	215.9, 262.5
1253	
1263	
1269	214.8, 253.5, 313.7
1279	
1281	
1283	
1299	212.4, 251.1, 305.4
1305	
1325	215.9, 271.1
1347	
1349	
1351	
1352	
1422	213.6, 255.8, 307.9
1453	
1456	
1533	
1535	
1653	
1738	
1747	
1766	
1870	
1958	
1973	

EXAMPLE 15

Non-Vindoline Containing Mutants

In the course of investigating alkaloid production in selected genetic lines and correlating observed alkaloid content with disease resistance, a mutation was discovered in a selected line (15453) that completely lacks monomeric vindoline. The trait is governed by a single recessive allele. Lines that express the mutation lack any detectable vindoline signal. Vindoline is a component of all known dimers, and is an expected component of trimers and polymers (as descried above). Leaves were subjected to alkaloid extraction as describe above. It was found that [illegible] the mutation lack all of these alkaloids. The lack [illegible] invention contain vindoline as a monomer. Vindoline-lacking plants thus also lacking dimers and

trimers/polymers) are especially sensitive to disease; they are also prone to infestation by mites, aphids and other common greenhouse pests. Keeping these plants alive in normal greenhouse conditions represents a significant challenge since opportune diseases and pests readily attack and kill the plants.

5

EXAMPLE 16

Assessment of Specific Chemical Bioactivity of Trimeric Alkaloids

Enriched trimer fractions, as described in Example 8 and as extracted using tartaric acid, and collection of specific chromatographic peaks yielding discreet HPLC eluates (specific 10 chemicals) according to the procedure described in Example 9 are analyzed for anti-fungal activity against cultures of *Phytophthora*, generally as described by Kato et al. (1996). The fractions or specific chemicals are applied to inert filter discs followed by evaporation of the solvent. The discs are then applied to axenic fungal or microbial cultures in petri dishes and incubated under conditions suitable for normal growth. The antifungal or antimicrobial activity of the individual 15 fractions or specific compounds is seen by measuring the zones of growth inhibition. It is found that the unsaturated trimeric or polymeric alkaloid compounds, e.g., compound 1279 possess antifungal activity.

EXAMPLE 17

20 Assessment of Specific Chemical Bioactivity of Trimeric Alkaloids

Purified alkaloids isolated from *Catharanthus* tissues are submitted to the National Cancer Institute (NCI) for determination of anti-cancer activity in their *in vitro* Anticancer Drug Discovery Screen (Boyd and Paull, 1995). Purified compound 1283 was tested by NCI, and as expected from its saturation, was not highly active in the screening assay. Purified compounds 1279 and 1281 25 are tested by NCI. Compound 1279 is found to be active in the screening assay and compound 1281 is found to have intermediate activity to that seen for 1279 and 1283.

It will be appreciated that the methods and compositions of the instant invention can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. It will be apparent to the artisan that other embodiments exist and do not depart from the spirit of

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